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Prevalence of *Bartonella henselae* and *Borrelia burgdorferi* Sensu Lato DNA in *Ixodes ricinus* Ticks in Europe▽

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***Bartonella* spp. can cause persistent bloodstream infections in humans and animals. To determine whether *Bartonella henselae* is present in questing *Ixodes ricinus* ticks, we analyzed the prevalence of *B. henselae* DNA among tick stages compared to the prevalence of DNA from *Borrelia burgdorferi* sensu lato, the pathogen most frequently transmitted by ticks. *B. henselae* DNA was present with a prevalence of up to ~40% in tick populations sampled in four European sites (Eberdingen, Germany; Klasdorf, Germany; Lembach, France; and Madeira, Portugal). The odds of detecting *B. henselae* DNA in nymphal ticks was ~14-fold higher than in adult ticks. No tick was found to be coinfected with *B. henselae* and *B. burgdorferi* sensu lato. Taken together, our data indicate that ticks might serve as a vector for the transmission of *B. henselae* to humans.**

In immunocompetent patients, *Bartonella henselae* infections often result in cat scratch disease (CSD), a self-limiting but often prolonged lymphadenitis; immunocompromised patients (e.g., AIDS patients) can suffer from vasculoproliferative disorders (bacillary angiomatosis, peliosis hepatis [1]). Cats are a confirmed reservoir host of *B. henselae* transmitting the pathogen by cat scratches or bites.

Several *Bartonella* species (e.g., *B. henselae*, *B. quintana*, and *B. vinsonii*) cause a persistent intraerythrocytic bacteremia in their respective mammalian reservoir hosts (7). *B. henselae* was detected in the peripheral blood of a wide range of mammals including domestic (e.g., cats, dogs, and horses) and wild animals (e.g., porpoise, lions, cheetahs, and wild felids). Obviously, such an asymptomatic, persistent bacteremia with *B. henselae* represents an important factor for the spread of the pathogens via blood-sucking arthropods. Mechanistic details determining the intraerythrocytic presence of *Bartonella* spp. have been investigated in detail in a *B. tribocorum* rat infection model mimicking Trench fever (a human disease caused by *B. quintana*); here, the pathogen persists several weeks in the circulating blood in an immunoprivileged intraerythrocytic niche (28).

Cat fleas are well established vectors for *B. henselae* (1). However, transmission by other arthropods, in particular ticks, has been suggested: *B. henselae* DNA was detected in questing *Ixodes pacificus* and *I. persulcatus* ticks in North America, Eastern Europe, and Russia, respectively (4, 13, 14, 22, 25) and in *I. ricinus* ticks feeding on people or domestic animals in Central Europe (24, 26). DNA of various *Bartonella* spp. has also been detected in keds, biting flies, and mites (reviewed in reference 2). Recently, ticks (*I. ricinus*) were experimentally infected with *B. henselae*. Inoculation of cats with salivary glands of infected ticks resulted in a *B. henselae* bacteremia (5). Nevertheless, controversial data about the prevalence of *Bartonella* spp. in ticks and their role as vectors for *B. henselae* exist (29).

Here, we present data on the prevalence of *B. henselae* and Lyme disease spirochetes in 654 questing ticks (*I. ricinus*) collected at four locations in Europe, suggesting that ticks might serve as potential vectors for the transmission of *B. henselae* to humans.

MATERIALS AND METHODS

Tick collection and identification. Questing nymphal and adult *I. ricinus* ticks were collected (i) at the edge of a forest in Eberdingen near Stuttgart (Germany), (ii) in an alder marsh outside the village Klasdorf located south of Berlin (Germany), (iii) near the town of Lembach in the northern Vosges region of France, and (iv) on pastured meadows on Madeira Island (Portugal) in 2004 by passing a flannel flag over the vegetation. Ticks were identified to stage and species by using microscopy and then preserved in 80% ethanol.

DNA extraction. 654 ticks were examined individually for the presence of DNA from *Bartonella* spp. and pathogenic *Borrelia* spp. (*Borrelia burgdorferi sensu lato*, including *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, and *B. spielmanii*). Each tick was dried by using a sterile filter paper, placed into a 1.5-ml tube, and crushed with a sterile pipette tip. DNA was extracted by using a QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany). To obtain higher DNA concentrations, the DNA was eluted twice with a reduced volume of AE buffer (100 μl instead of 200 μl).

Detection of *Bartonella* spp. and *B. burgdorferi* sensu lato DNA in questing

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TABLE 1. Primer designation and sequences used for the detection of *Bartonella* spp. and *B. burgdorferi* sensu lato from *I. ricinus* ticks

Target	Amplicon size (bp)	Primer		Reference
		Designation	Sequence	
Bartonella sp. 16S rDNA (first round)	1,210	A-proteo r-Alpha-sh	AGA GTT TGA TC/AC TGG CTC AGA GTA GCA CGT GTG TAG CCC A	6
Bartonella sp. 16S rDNA (second round)	990	Bart r-BH	CAC TCT TTT AGA GTG AGC GGC AA CCC CCT AGA GTG CCC AAC CA	6
Bartonella 16S-23S ITS region	775	325s 1100as	CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG GAA CCG ACG ACC CCC TGC TTG CAA AGC A	19
B. burgdorferi sensu lato ospA	496	$OspA_{outer}$fw $OspA_{outer}$rev	GGT CTA ATA TTA GCC TTA ATA GGC ATG TCA GCA GCT AGA GTT CCT TCA AG	9
B. burgdorferi sensu lato ospA	398	$OspA_{inner}$fw $OspA_{outer}$rev	CAT GTA AGC AAA ATG TTA GCA GCC CTG TGT ATT CAA GTC TGG TTC C	9
Tick mitochondrial 16S rDNA	490	MT16SA MT16SB	CGC CTG TTT ATC AAA AAC AT CTC CGG TTT GAA CTC AGA TC	21

ticks. PCRs (*Bartonella* spp. 16S rDNA, *Borrelia burgdorferi sensu lato ospA*, tick mitochondrial 16S rDNA) were performed in the routine diagnostic laboratories of the Institute of Medical Microbiology and Hygiene at the University Hospital of Tuebingen (Germany). These laboratories are certified according to the DIN EN ISO/IEC 17025 and 15189 standards (laboratory identification code: DAC-ML-0159-02-10). During the time of this study, no increase in the number of *Bartonella*-positive PCRs in routine samples was reported making a possibility of DNA cross-contaminations highly unlikely.

For the detection of *B. henselae* DNA a nested PCR specific for the 16S ribosomal DNA (rDNA) (6) was used, and for the detection of *B. burgdorferi sensu lato* DNA a PCR specific for the *ospA* gene (9) was used (limit of detection, ~100 DNA equivalents; Table 1). Each reaction was performed in a volume of 25 μl containing 0.5 μl (25 μM) of each primer (Metabion, Martinsried, Germany), 0.5 μl of deoxynucleoside triphosphate (10 mM each), 2.5 μl of 10× PCR buffer, 0.5 U of *Taq* DNA polymerase (Roche, Mannheim, Germany), and 2.5 μl of the DNA extract in the first round or 0.5 μl of the amplification product in the second round of the nested PCR. In each PCR, a negative (sterile water) and a positive control (PCR-amplified target-DNA blunt-end cloned into a pTOPO vector [Invitrogen, Hildesheim, Germany]) were processed in parallel.

To confirm that DNA was extractable and amplifiable from ticks stored in ethanol, a fragment of the tick mitochondrial 16S rDNA was amplified in random samples (data not shown) as described previously (21).

Sequencing and sequence analysis. All amplified fragments obtained using *Bartonella* spp.-specific primers and five fragments obtained using *Borrelia burgdorferi sensu lato* specific primers were sequenced on an ABI 3130 XL sequencer (4Base Lab; ABI, Reutlingen, Germany). Sequences were analyzed by using the BLAST search algorithm of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Differentiation of *Bartonella* DNA by using 16S-23S gene ITS PCR. For a subset of PCR-positive ticks, intergenic transcribed spacer (ITS) analysis was performed at the Intracellular Pathogens Research Laboratory and the Center for Comparative Medicine and Translational Research, College of Veterinary Medicine, North Carolina State University, Raleigh, NC. For this purpose, PCR screening of the *Bartonella* ITS region was performed as described previously (15).

Statistics. The presence of *B. henselae* and *B. burgdorferi sensu lato* was analyzed for systematic differences between nymphal and adult ticks while adjusting for the location in a logistic regression. For χ^2 tests the degrees of freedom (df) and *P* values are given. 95% confidence intervals (CI) were calculated for prevalences and for odds ratios of prevalences using the software JMP 7.0.2 (SAS Institute, Heidelberg, Germany). The hypothesis of independence of both infections was tested while stratifying for location and stage (nymph or adult), assuming independence of subgroups. The exact one-sided *P* value was computed as the product of binomial cumulative probabilities of the observed number (zero) of coinfections, given the expected probabilities computed from the data, using R2.5.0 software (The R Foundation for Statistical Computing).

RESULTS

Prevalence of *B. henselae* DNA in ticks collected in Europe. Of 654 questing ticks (419 adults, 235 nymphs), 84 ticks (12.8%) contained *B. henselae* DNA detected by 16S rDNA nested PCR and sequence analysis (Table 2). Remarkably, no other *Bartonella* species were detected.

The highest prevalence of *B. henselae* DNA was observed in nymphs collected in Lembach, France (38.2%), followed by nymphs collected on Madeira Island, Portugal (32.3%) and Eberdingen, Germany (11.8%). No nymphs were collected in Klasdorf, Germany. In adult ticks, *B. henselae* DNA was most

TABLE 2. Frequency of detection of *B. henselae* and *B. burgdorferi* sensu lato DNA in European ticks

Collection site (coordinates)	No. of positive ticks/no. of ticks examined (%)			
	B. henselae		*B. burgdorferi* sensu lato	
	Nymphs	Adults	Nymphs	Adults
Eberdingen, Germany (48°52′51″N, 08°57′51″O)	4/34 (11.8)	1/199 (0.5)	4/34 (11.8)	44/199 (22.1)
Klasdorf, Germany (52°01′31″N, 13°33′ 29″O)	ND[a]	2/70 (2.9)	ND	3/70 (4.3)
Lembach, France (49°00′30″N, 07°47′ 23″O)	39/102 (38.2)	6/50 (12.0)	2/102 (2.0)	0/50 (0.0)
Madeira, Portugal (32°42′35″N, 16°54′ 01″W)	32/99 (32.3)	0/100 (0.0)	0/99 (0.0)	0/100 (0.0)
Total	75/235 (31.9)	9/419 (2.1)	6/235 (2.6)	47/419 (11.2)

[a] ND, not done; no nymphs were collected in Klasdorf.

prevalent in Lembach (12.0%), followed by Klasdorf (2.9%) and Eberdingen (0.5%). In Madeira, none of the 100 examined adult ticks contained *B. henselae* DNA. Overall, ~15 times more nymphs (31.9%) contained *B. henselae* DNA than did adults (2.1%).

Six *B. henselae* DNA samples originating from ticks collected in Lembach were analyzed by sequencing the 16S-23S rRNA ITS region. In at least four of six specimens the detected DNA showed 100% homology (548/548 bp) compared to those of *B. henselae* strains URBHLLY8 and URBHLIE9 (GenBank accession numbers AF312495 and AF312496) which were originally isolated in Marseille, France, in 1996 (8). DNA sequences from two further ticks showed only a one-base mismatch (547 of 548 bp) with *B. henselae* strain URBHLLY8 and URBHLIE9 and, therefore, also represent most probably ITS sequences of these two strains. The data were confirmed by sequence analysis of the bacteriophage-associated heme-binding protein gene (Pap31; results not shown).

Prevalence of *B. burgdorferi sensu lato* DNA in ticks collected in Europe. The prevalence of *B. burgdorferi sensu lato* DNA was assessed as an additional internal control. Of 654 questing ticks analyzed, 53 (8.1%) contained DNA of *B. burgdorferi sensu lato* (see Table 2). Five amplicons were sequenced, confirming the identity of *B. burgdorferi sensu lato* (data not shown). In nymphs (collected at Eberdingen and Lembach), *B. burgdorferi sensu lato* DNA was detected in 4.4% (Eberdingen, 11.8%; Lembach, 2.0%). In adult ticks (collected at Eberdingen, Klasdorf, and Lembach) the prevalence of pathogenic spirochetes was ~3 times higher (14.7%) than in nymphs. In Madeira, no positive samples were detectable. In no tick (out of 654) was a coinfection with *B. henselae* and *B. burgdorferi sensu lato* observed.

Statistical analysis of the prevalences of *B. henselae* and *B. burgdorferi* DNA. Comparing *B. henselae* and *B. burgdorferi sensu lato* infection rates in nymphs and adult ticks, the raw odds ratios of DNA detection in nymphs relative to that in adult ticks were 1/3 for *B. henselae* and 28 for *B. burgdorferi sensu lato*. Since no nymphs were collected in Klasdorf, only 584 specimens from Eberdingen, Lembach, and Madeira were analyzed by logistic regression. The odds of detecting *B. henselae* DNA in nymphs was 13.8-fold (CI = 6.5 to 34 times) the odds of detecting it in adult ticks (χ^2 with 1 df was 63, $P = 1.9 \times 10^{-15}$) while adjusting for the fact that the prevalence of *B. henselae* DNA differed from place to place (χ^2 with 2 df was 17.6, $P = 0.00015$). Neither the 99 nymphs, nor the 100 adult ticks collected on Madeira harbored *B. burgdorferi sensu lato* DNA. Therefore, the one-sided 95% CI for the prevalence has upper limits of 2.7 and 2.6%. With 385 specimens from Eberdingen and Lembach, the odds of detecting *B. burgdorferi sensu lato* DNA in nymphs was 0.61-fold (CI = 0.21 to 1.49 times), and the odds of detecting it in adult ticks (χ^2 with 1 df) was 1.13 ($P = 0.29$), while adjusting for the fact that the prevalences exhibited an odds ratio between Eberdingen and Lembach of 15 (χ^2 with 1 df was 24.2, $P = 8.5 \times 10^{-7}$). When testing independence of the two infections, only combinations of place and stage with positive numbers of infected ticks contribute to the *P* value, excluding samples from Madeira (where *B. burgdorferi* appeared absent) and the adult ticks from Lembach. The *P* value of 0.21 suggests no significant deviation from independence, either in the total of 405 ticks or in the four subgroups.

DISCUSSION

Bloodstream infections are a common feature of human infections with *Bartonella* spp. such as, *B. henselae*, *B. quintana*, and *B. bacilliformis* (7). Symptoms range from mild conditions to severe life-threatening or fatal infections (1). Domestic cats and cat fleas are the most frequent source of *B. henselae* infections in humans; dogs may also serve as a reservoir for *B. henselae* (16). The biphasic Carrion's disease is caused by *B. bacilliformis*, which is endemic in some areas of the Andes (Peru). The acute phase, called Oroya fever, is characterized by an intraerythrocytic bacteremia that often results in a fatal hemolytic anemia; humans are the only known reservoir host for *B. bacilliformis* (1). While the hemolytic activity of *B. bacilliformis* seems to be unique among *Bartonella* spp., prolonged periods of intracellular erythrocyte parasitism appear to be crucial for the pathogenicity of the genus *Bartonella*.

Due to the ability of *Bartonella* spp. to infect and reside within erythrocytes of a constantly growing number of known animal hosts, they might be transmitted by a variety of other arthropod vectors. A potential transmission of *B. henselae* to humans via ticks is underlined by the observations that *B. henselae* has been detected in *Ixodes ricinus* ticks collected from humans (26). It was reported from the United States that tick exposure was related to *B. henselae* infections (11). Californian *I. pacificus* ticks harbored DNA of *B. quintana*, *B. henselae*, *B. washoensis*, and *B. vinsonii berkhoffii* (4, 14). Furthermore, tick exposure was not only found to be a risk factor for CSD in humans but also for *B. vinsonii berkhoffii* seropositivity in dogs (23). More than a third of adult Russian *I. persulcatus* ticks were infected by *B. henselae* (22, 25). In France and The Netherlands, surveys of questing *I. ricinus* demonstrated the presence of *Bartonella* DNA in ticks; those that were characterized further appeared to be species associated with ungulates, such as *B. schoenbuchensis* and *B. capreoli* (3, 12, 27). All this strongly suggests that ticks might serve as a vector for *Bartonella* spp.

I. ricinus ticks most frequently transmit Lyme disease spirochetes. Larvae acquire spirochetes from an infected host and transmit the bacteria during subsequent blood meals as nymphs; they may also acquire spirochetes in their nymphal stage and transmit the bacteria as adults. Generally, questing adult ticks are more frequently infected by Lyme disease spirochetes than nymphal ticks since they had two opportunities to acquire the pathogen if feeding on competent hosts. Interestingly, we found that the prevalence ratio of *B. henselae* DNA in nymphal and adult ticks differs significantly from that of *B. burgdorferi sensu lato* and that the difference of harboring *B. henselae* DNA was always high between nymphs and adult ticks (odds ratio of ~14), suggesting that this pathogen may differ in its host associations from *B. burgdorferi*. A possible explanation might be different host preferences of larvae and nymphs: for instance, on pheasants and lizards, nymphs appear to feed more frequently than larvae do and vice versa for small rodent hosts (17, 18, 20). It may be that hosts on which larvae preferably feed are permissive for *B. henselae*, but those on which nymphs feed most frequently fail to support the patho-

gen. Indeed, *B. henselae* has been isolated from wood mice, *Apodemus sylvaticus* (10), a common host of larval *I. ricinus* ticks, although this observation was controversially discussed at the 6th *Bartonella* Meeting in Chester, United Kingdom (in June 2009).

It is questionable whether the detection of *B. henselae* DNA in ticks is the equivalent of detecting viable bacteria. To verify vector competence of *I. ricinus*, acquisition and subsequent transmission of this pathogen from and to a competent reservoir host needs to be demonstrated. In a first approach, various stages of *I. ricinus* ticks were fed on *B. henselae*-infected blood through an artificial feeding membrane, and pathogen DNA was subsequently detected in the carcasses of the molted ticks but not in their salivary glands (5). Only upon refeeding on uninfected blood did their salivary glands contain viable and infective *B. henselae*; the pathogen was also detectable in the blood on which the ticks had fed. Although this *in vitro* setting cannot unambiguously prove natural pathogen transmission between host and tick, it demonstrates that *B. henselae* may be passed trans-stadially during tick development and infect the salivary glands of ticks. Both observations are prerequisite factors for vector competence. Finally, and despite of their low number ($n = 6$), the results of 16S-23S ITS region sequence analysis underline the need for further investigations to prove whether the *B. henselae* strains URBHLLY8 and URBHLIE9 (originally isolated from two patients suffering from CSD and endocarditis, respectively [8]) are prevalent or even endemic in tick populations.

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